IJSM International Journal of Secondary Metabolite

Int. J. Sec. Metabolite, Vol. 4: 3, (2017) pp. 27-36

Special Issue 1: Research Article

ISSN: 2148-6905 online Journal homepage: http://www.ijate.net/index.php/ijsm

Investigation of Relationship Between Chemical Stress Factors and Certain Metabolites Including Cardenolides in Callus Cultures of Endemic Turkish *Digitalis* L. Species

Gunce SAHİN[*1], Sandeep Kumar VERMA[2], Ekrem GUREL[1]

[1]Abant Izzet Baysal University, Faculty of Science and Art, Department of Biology, 14280, Bolu, Turkey
[2]Department of Horticulture, Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 66071, South Korea

Received: 28 April 2017 - Accepted: 04 June 2017

Abstract: The aim of the present research is to obtain relationship between different stress treatments [Cu (copper) and Hg (mercury)] and content of cardiac glycosides (digoxigenin, gitoxigenin, lanatoside C, digoxin and digitoxin) as secondary metabolites of commercial value for the pharmaceutical industry and to determine the antioxidant metabolites against stress conditions in callus cultures of endemic Turkish *Digitalis* species. The effects of different stress treatments on cardiotonic glycoside accumulation in *D. lamarckii* Ivanina, *D. trojana* Ivanina, *D. davisiana* Heywood and *D. cariensis* Boiss. ex Jaub. et Spach were investigated using HPLC. HPLC analysis revealed that all stress conditions were significantly effective at 5% significance level according to their control groups. The predominant cardiac glycoside was lanatoside C (Lan C) followed by digitoxin, digoxigenin, gitoxigenin and digoxin. No digoxin was detected in all treatments as well as in control groups. For the calibration curves, concentrations of 5, 10, 20, 30 and 40 mg/l digoxigenin, gitoxigenin, lanatoside C, digoxin and digitoxin were used (R^2= 0.99). Cardenolides were eluted with acetonitrile (A) and water (B) gradients as follows: 0 to 20 min 20% (A), 80% (B); 20 to 23.40 min 30% (A), 70% (B); 23.40 to 30 min 25% (A), 75% (B) and 30 to 40 min 40% (A), 60%(B). Average peak area of the glycoside in samples was automatically calculated and monitored by ChemStation LC/MS software against that of standards. Enhanced production of cardenolides was achieved from callus cultures elicited with 50 µm $CuSO_4$ and $HgCl_2$. Higher amounts of cardenolides were obtained when callus of four *Digitalis* species were elicited with CuSO4. Results demonstrated that catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1) activities, the total contents of phenolics and proline were markedly stimulated under stress conditions. All these results indicated that treatments have induced changes in the redox state of callus cells and suggest that this alteration change cardenolides accumulation and antioxidative status in *Digitalis* L. callus cultures.

Keywords: Antioxidant, cardiac glycosides, *Digitalis* L., heavy metal stress

1. INTRODUCTION

Digitalis L. produces various cardiac glycosides which have potential to treat many diseases such as edema, myocardial infarction, arterial hypertension, cardiac dysfunction, angina and hyertropy [1]. Besides their cardiotonic effects, these compounds are also effective chemotherapeutic agents, especially in breast and prostate cancer treatments [2]. *Digitalis* species is distributed in the Mediterranean region, Western Asia and Europe. *Digitalis davisiana* Heywood, *Digitalis lamarckii* Ivanina, *Digitalis cariensis* Boiss. ex Jaub. et Spach

[*]Corresponding Author E-mail: guncesahin@gmail.com

ISSN: 2148-6905 online /© 2017 DOI: 10.21448/ijsm.356249

and *Digitalis trojana* Ivanina are endemic to Turkey [3]. Traditionally, cell and organ cultures have been used for production of secondary metabolites, but the yield of cardiac glycosides have been low. Attempts have been made to increase concentration of metabolites in shorter period of time. Exogenous addition of biotic and abiotic elicitors is considered to be one of the most promising strategies for the induction of secondary metabolites production [4]. Zhao et al. [5] proposed that these elicitor molecules act as extracellular or intracellular signals and initiate a signal transduction network that is required for the activation of transcription factors, which organize the expression of genes included in plant secondary metabolism [6]. Among the various abiotic elicitors, heavy metal stresses have been considered as effective elicitors for the increased production of secondary metabolites in *in vitro* cultures. Heavy metal stress has become a headmost environmental threat to crop production. Being a potential hazardous factor, toxic metals decrease the plant growth, yield and sustainability of production, thus can cause the alarming situation for food availability. Plants under the stress environment facing the alterations of cellular protein functions, lipid and thylakoid structures. Disturbance or breakage of these structures is directly linked with plant photosystem that can affect the senescence process [7,8]. Copper (Cu), microelement, has important physiological functions in plants. At higher concentrations, it leads to physiological and morphological disturbances, as a consequence decrease the yield [9]. Mercury (Hg) is a toxic metal that can be absorbed from the atmosphere and soil. It can be accumulate in the plant organs and cause to phytotoxic effects. The toxic metal threshold level in the tissue is defined by the 'stress point'. Beyond the stress point, the cell will be irreversibly damaged [10]. These metabolic changes can directly trigger the plant defense system, including enzymatic and non-enzymatic antioxidants to cope with overproduce of reactive oxygen species (ROS) in the cell [11]. As a consequence of unfavorable conditions, activation of genes related to the enzymatic defense system, including catalase (CAT), superoxide dismutase (SOD), guaicol peroxidase (POD), glutathione reductase (GR) and the non-enzymatic antioxidants such as glutathione, ascorbate (vitamin C), carotenoids, α-tocopherol (vitamin E), proline and various phenylpropanoid derivatives (phenolic compounds) were observed [12]. On the basis of background information, the aim of this work is to investigate the induction of cardenolide compounds (lanatoside C, digitoxin, digoxigenin, gitoxigenin and digoxin) in *D. davisiana* Heywood, *D. lamarckii* Ivanina, *D. trojana* Ivanina and *D. cariensis* Boiss. ex Jaub. et Spach, in response to application of heavy metal toxicity and to determine the correlation between the cardiotonic glycosides accumulation and stress responsive antioxidant defense system.

2. MATERIAL and METHODS

2.1. Plant materials

Seeds of four endemic Digitalis species (*D. davisiana* Heywood, *D. lamarckii* Ivanina, *D. trojana* Ivanina and *D. cariensis* Boiss. ex Jaub. et Spach) were collected in August to September, 2010. Seeds of *D. davisiana* and *D. cariensis* from Alanya-Mahmutlar (N360 31.916', E032014.402') (N360 30.767', E032012.695', 03.09.2010), seeds of *D. lamarckii* around the Ankara-Kızılcahamam (N40037.709', E032026.265') and *D. trojona* were collected from the National Park of Ida Mountains (N390 38.885', E0260 57.402').

2.2. Experimental Design

The seeds were cultured on MS medium including containing 3% sucrose and 0.8% agar (pH 5.7-5.8) for germination. Hypocotyl explants were cultured on MS medium including vitamins (0.5 ppm TDZ and 0.25 ppm IAA) (Sahin et al., 2013) for callus induction. After 30 days of culture 50 µm $HgCl_2$ and 50 µm $CuSO_4$ were used in cultures in order to expose the callus cultures to chemical stress for 10 days [13].

2.3. Extraction and HPLC analysis of cardenolides

Cardenolide extraction was determined according to the modified method of Wiegrebe and Witchtl [14]. Qualitative and quantitative analysis of cardenolides were detected and calculated as previously described HPLC protocol [15].

2.4. Enzyme extraction, protein determination and assays of enzymes

Callus material was homogenized as previously described protocol [15], then tissue extracts were kept at -80 ^{o}C for determination of superoxide dismutase (SOD, EC 1.15.1.1) [16] and catalase (CAT, EC 1.11.1.6) activity [17]. The protein content was determined according to Lowry method [18].

2.5. Total phenolic assay

The total phenolic content were determined using the Folin-Ciocalteau [19] method. The total phenolic content of extracts was expressed as mg gallic acid equivalents (GAE)/ g dw.

2.6. Proline analysis

Proline was determined according to the method of Bates et al., [20]. Proline content was calculated as µmol proline /g dw.

2.7. Statiscal Analysis

Data were statistically analyzed using SPSS Version 15.0 (SPSS Inc., Chicago, IL, USA) and Duncan's multiple range test at $P \leq 0.05$. Each treatment was made in triplicate.

3. RESULTS

Callus was initiated from hypocotyl explants cultured on MS medium supplemented with 0.25 mg L^{-1} IAA and 0.5 mg L^{-1} TDZ. After 30 days, callus was transferred to MS medium containing 50 µm $HgCl_2$ and 50 µm $CuSO_4$ for 10 days. Here, we examined the effects of $HgCl_2$ and $CuSO_4$ as an heavy metal stress factors on cultures which induced cardiotonic glycoside accumulation and antioxidant activities in four *Digitalis* species (*D. cariensis, D. davisiana, D. trojana, D. lamarckii*). Application of $HgCl_2$ and $CuSO_4$ to the medium were not affected cell viability. However, the colour of the callus changed from green (control group) to brownish with $HgCl_2$ and $CuSO_4$ applications (Figure 1). Results related to cardenolides accumulation of treated and untreated plants of four *Digitalis* species (*D. davisiana Heywood, D. lamarckii Ivanina, D. trojana Ivanina* and *D. cariensis* Boiss. ex Jaub. et Spach) are shown in Table 1.

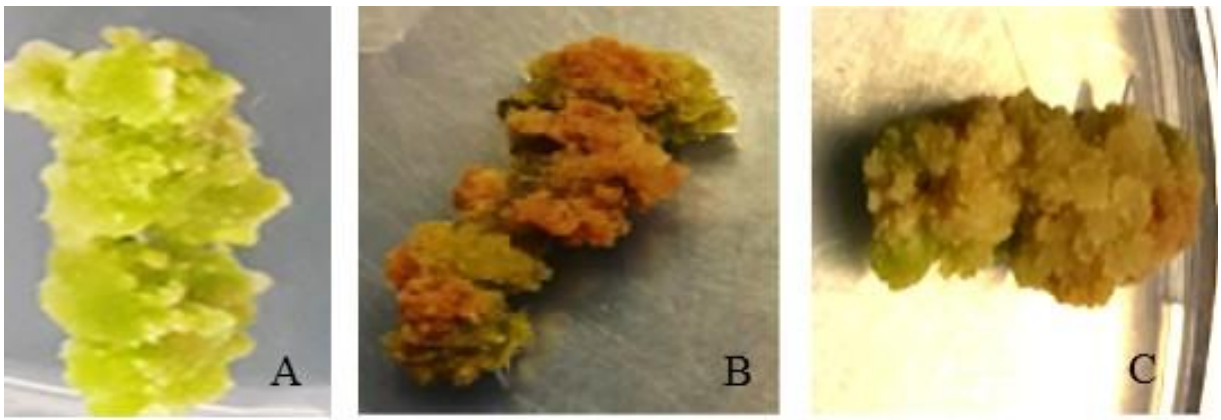

Figure 1. Effects of culture media and stress factors on morphological characters of induced callus: (A-C): (A): Callus growth in MS medium+0.25 mg L^{-1} IAA and 0.5 mg L^{-1} TDZ (control group), (B): Callus growth in MS+50 µm $HgCl_2$, (C): Callus growth in MS+50 µm $CuSO_4$

Table 1. Results of cardenolides accumulation in the control and elicited callus tissues of *Digitalis* species. Means followed by different letters in the same column are significantly different ($P < 0.05$).

Species	Treatments	Amount of cardenolides (μg g^{-1}, dw)				
		Digoxigenin	Gitoxigenin	Lan C	Digoxin	Digitoxin
D. lamarckii	Control	9.04^{h}±0.62	6.46^{def}±0.36	302.04^{i}±7.49	<LOD	8.67^{d}±0.49
	$CuSO_4$	11.41^{ef}±1.96	6.41^{ef}±0.51	721.80^{c}±8.78	<LOD	11.83^{bc}±3.78
	$HgCl_2$	10.37^{fgh}±0.41	6.49^{ef}±0.57	377.23^{g}±5.20	<LOD	9.27^{cd}±2.08
D. trojana	Control	12.96^{de}±0.56	6.12^{fg}±0.13	285.63^{j}±2.57	<LOD	7.48^{d}±0.50
	$CuSO_4$	17.13^{b}±1.31	7.62^{cd}±1.13	619.83^{e}±7.23	<LOD	9.66^{cd}±0.77
	$HgCl_2$	12.49^{de}±1.10	6.61^{def}±0.83	346.37^{h}±9.47	<LOD	8.68^{d}±0.77
D. davisiana	Control	9.56^{gh}±0.10	5.16^{g}±0.20	259.85^{k}±8.17	<LOD	7.55^{d}±0.50
	$CuSO_4$	18.99^{a}±0.17	7.75^{c}±0.21	844.097^{b}±7.89	<LOD	13.52^{b}±0.93
	$HgCl_2$	15.44^{c}±0.77	7.34^{cde}±0.33	461.13^{f}±6.08	<LOD	9.33^{cd}±0.31
D. cariensis	Control	10.73^{fg}±0.17	8.33^{c}±0.47	280.71^{j}±7.14	<LOD	9.06^{d}±0.27
	$CuSO_4$	14.09^{cd}±0.59	12.16^{a}±0.63	939.21^{a}±9.09	<LOD	16.13^{a}±0.54
	$HgCl_2$	13.07^{d}±0.43	10.01^{b}±0.67	673.23^{d}±6.58	<LOD	13.92^{ab}±0.16

Note: LOD; limit of detection.

Addition of $HgCl_2$ and $CuSO_4$ (50 μm) into media significantly affected the cardenolides accumulation as compared to control. Especially, $CuSO_4$ treatments played a pivotal role to the accumulation of cardenolides. Lanatoside C was the predominant cardiac glycoside followed by digoxigenin, digitoxin, gitoxigenin and digoxin. On the other hand, the content of digoxin was below the limit of detection in all treatments. The use of $CuSO_4$ and $HgCl_2$ led to a drastic increase in the accumulation of Lan C in all *Digitalis* species. The control (non-treated) callus produced 302.04±7.49 μg/g dw Lan C while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 721.80±8.78 μg/g dw and 377.23±5.20 μg/g dw Lan C respectively in *D. lamarckii* callus cultures. Similar to *D. lamarckii*, the positive correlation between the accumulation of Lan C and $CuSO_4$ - $HgCl_2$ applications was detected in *D. trojana* callus cultures. 285.63±2.57 μg/g dw Lan C was found in the control (non-treated) callus while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 619.83±7.23 μg/g dw and 346.37±9.47 μg/g dw Lan C, respectively. In *D. davisiana* cultures, the accumulation of Lan C was significantly induced as a concequence of $CuSO_4$ and $HgCl_2$. Lan C of control was 259.85±8.17 μg/g dw while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 844.097±7.89 μg/g dw and 461.13±6.08 μg/g dw Lan C, respectively. CuSO4 as well as $HgCl_2$ stress was followed by a significantly enhanced accumulation of Lan C in *D. cariensis* callus cultures. The control (non-treated) callus produced 280.71±7.14 μg/g dw Lan C while those cultured on under chemical stress by $CuSO_4$ and $HgCl_2$ producing 939.21±9.09 μg/g dw and 673.23±6.58 μg/g dw Lan C respectively. Although a noticeable increase was observed in digitoxin, digoxigenin content under the exposure of $CuSO_4$, there was not any significant increase in gitoxigenin, digitoxin, digoxigenin content under the exposure of $HgCl_2$ compared to non-treated callus in *D. lamarckii*. In *D. trojana* cultures, digoxigenin (17.13±1.31 μg/g dw) and gitoxigenin levels (7.62±1.13 μg/g dw) were significantly increased under $CuSO_4$ stress. But it is apparent that, at 5% significance level, there was not any significant change observed between control groups and digitoxin content under both applied treatments. Addition of $CuSO_4$ and $HgCl_2$ into the medium significantly increased the digoxigenin, gitoxigenin and digitoxin content in *D. davisiana* and *D. cariensis* callus cultures as compared with respective controls. The data regarding the antioxidant and non-enzymatic antioxidant enzymes are presented in

Table 2. Activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) were significantly enhanced in all species under 50 µm $CuSO_4$ and $HgCl_2$ treatments as compared with respective controls.

Table 2. Effect of different treatments of heavy metals on enzymatic and non-enzymatic antioxidant activities in the callus cultures of *Digitalis* species.

Species	Treatments	SOD	CAT	Total phenolic	Proline
D. lamarckii	Control	$0.14^{g}\pm0.04$	$19.30^{d}\pm0.70$	$154.45^{k}\pm0.72$	$0.76^{j}\pm0.006$
	$CuSO_4$	$0.55^{de}\pm0.07$	$28.61^{a}\pm1.20$	$196.12^{i}\pm1.25$	$1.33^{e}\pm0.006$
	$HgCl_2$	$0.31^{f}\pm0.03$	$21.60^{c}\pm0.94$	$174.87^{j}\pm0.72$	$1.23^{g}\pm0.012$
D. trojana	Control	$0.16^{g}\pm0.05$	$15.55^{f}\pm0.98$	$219.46^{g}\pm0.72$	$0.72^{k}\pm0.010$
	$CuSO_4$	$0.46^{e}\pm0.08$	$23.50^{b}\pm1.31$	$276.54^{d}\pm1.90$	$1.29^{f}\pm0.006$
	$HgCl_2$	$0.36^{f}\pm0.09$	$21.85^{c}\pm1.41$	$238.20^{f}\pm1.44$	$1.13^{h}\pm0.006$
D. davisiana	Control	$0.48^{e}\pm0.05$	$11.45^{gh}\pm0.59$	$198.62^{h}\pm1.76$	$0.76^{j}\pm0.006$
	$CuSO_4$	$0.88^{b}\pm0.04$	$17.68^{e}\pm1.05$	$371.95^{b}\pm0.88$	$1.68^{b}\pm0.012$
	$HgCl_2$	$0.78^{c}\pm0.02$	$14.82^{f}\pm0.44$	$277.37^{d}\pm1.76$	$1.46^{d}\pm0.010$
D. cariensis	Control	$0.46^{e}\pm0.03$	$8.24^{i}\pm0.93$	$252.38^{e}\pm1.25$	$0.81^{i}\pm0.006$
	$CuSO_4$	$1.14^{a}\pm0.01$	$12.66^{g}\pm0.63$	$476.95^{a}\pm0.72$	$1.82^{a}\pm0.006$
	$HgCl_2$	$0.64^{d}\pm0.05$	$10.69^{hi}\pm0.63$	$330.29^{c}\pm1.90$	$1.66^{c}\pm0.010$

Note: Values followed by same small letters are not significantly different at $P<0.05$.

The CAT activity in control group ranged from 8.24 µmol H_2O_2/min/mg protein for *D. cariensis* to 19.30 µmol H_2O_2/min/mg protein for *D. lamarckii* followed by 15.55 µmol H_2O_2/min/mg protein for *D. trojana*. When incubating callus with 50 µm $CuSO_4$ induced CAT activity ranged from 12.66 µmol H_2O_2/min/mg protein for *D. cariensis* to 28.61 µmol H_2O_2/min/mg protein for *D. lamarckii* followed by 23.50 µmol H_2O_2/min/mg protein for D. trojana. Addition of 50 µm $HgCl_2$ to the media also enhanced CAT activity ranged from 10.69 µmol H_2O_2/min/mg protein for *D. cariensis* to 28.61 µmol H_2O_2/min/mg protein for *D. lamarckii*. Exposure of heavy metals was followed by a remarkable increase in SOD activity as compared to the control groups. In control, SOD activity ranged from 0.14 U/mg protein for *D. lamarckii* to 0.48 U/mg protein for *D. davisiana* followed by 0.46 U/mg protein for *D. cariensis*. While that cultured expose with $CuSO_4$ induced SOD activity ranged from 0.55 U/mg protein for *D.lamarckii* to 1.14 U/mg protein for *D. cariensis*. Exogenously applied $HgCl_2$ induced SOD activity ranged from 0.31 U/mg protein for *D.lamarckii* to 0.78 U/mg protein for *D. davisiana.* Besides the enzymatic defense machinery, we also studied non-enzymatic antioxidant response under different treatments of heavy metals. Non-enzymatic antioxidant named as total phenolic and proline levels were increased in all species under $CuSO_4$ and $HgCl_2$ stress as compared to control. Maximum total phenolic and proline production were determined as 476.95 µg GA mg^{-1} (1.88 -fold higher than control) and 1.82 µmol g^{-1} (2.24- fold higher than control) with the elicitation of $CuSO_4$ respectively.

4. DISCUSSION

Plant secondary metabolism is the source for many fine chemicals of commercial importance. One group of natural products of major interest in the pharmaceutical industry is cardiac glycosides from *Digitalis* species. Levels of plant carbon-based secondary compounds are partly under genetic control and determined in part by environmental conditions [21] therefore, in order to maximise the production of a specific natural product, it will be necessary to understand the various factors that control and influence its biosynthesis. In the case of

Digitalis plants, previous studies have reported that cardenolide biosynthesis is basically dependent on morphological differentiation [22] and genotype [23], although numerous environmental factors may determine, in a greater or lesser degree, plant productivity. Thus, it is known the influence that mineral nutrients [24, 25], CO_2 and water stress [26], and light conditions [27] exert on cardenolide accumulation. Moreover, in our previous papers, we showed that H_2O_2 pre-treatment [15] and elimination of Ca, Mg or both from the medium [28] resulted in an increase in cardenolides, enzymatic and non-enzymatic antioxidants in callus cultures of *Digitalis davisiana* Heywood, *Digitalis lamarckii* Ivanina, *Digitalis trojana* Ivanina and *Digitalis cariensis* Boiss. ex Jaub. et Spach. Elicitation strategies have been widely used to induce the production of secondary metabolites in *in vitro* cell cultures [29]. In the light of earlier studies, it was concluded that H_2O_2 increase occurred after Cu, Cd [30] and Hg [13] treatment of *A. thaliana* and *S. lycopersicum*, respectively. Similarly, Smith et al. [31] showed that production of umbelliferone, which is a phytoalexin produced in response to stress or infection in whole plants, was stimulated in suspension cultures of *Ipomoea batatas* (L.) Poir. using $HgCl_2$. Korsangruang et al. [32] found that $CuSO_4$ enhanced the accumulation of isoflavonoid compounds in *Pueraria candollei* cell suspension culture. However, there are not any reports of *Digitalis* tissue cultures in which improvements in cardenolide production have been achieved by heavy metal treatments.

The more recent identification and characterization of several enzymes/genes involved in pregnane and cardenolide metabolism, such as 3β-hydroxysteroid dehydrogenase and progesterone 5β-reductase. P5βR is considered to be a key enzyme in cardenolide biosynthesis as: it is the first stereospecific enzyme of the pathway leading to 5β-configured derivatives; it appears to be the initial step in cardenolide biosynthesis. Pérez-Bermúdez et al. [33] indicated that P5βR2 is a critical component for the chemical defense of foxglove plants against herbivores, through cardenolide accumulation, in association with ethylene and H_2O_2 signaling in *Digitalis purpurea*. Available information suggests that H_2O_2 directly regulates the expression of numerous genes involved in plant defense and the related pathways such as antioxidant enzymes, defense proteins and transcription factors [34]. In our studies, heavy metal stress significantly induced cardenolide production in callus cultures of all *Digitalis* species. The increase in secondary metabolite concentrations in the callus cultures under heavy metal stress may also be associated with the alterations in the activity of P5βR2 gene related to cardenolides. This was probably due to the reason that heavy metal-induced ROS generation was responsible for transcriptional activation of genes encoding enzymes involved in cardenolide biosynthesis. Although, the applications of heavy metals significantly increased the cardenolides accumulation in all *Digitalis* species, there was no digoxin detected in all treatments. It is well known that digilanidase enzyme catalyzed deglucosylation and subsequent deacetylation of Lan C to make into digoxin in the leaves [35]. This was probably due to the reason that the amount of digoxin in the callus tissues examined was found to be below the detection limit of the determination used.

In current work, CAT and SOD activity were significantly increased with the $CuSO_4$ and $HgCl_2$. According to Romero-Puertas et al. [36], many heavy metals could result in increased activity of NADPH oxidase partially related with O_2^- formation. Correspondingly, heavy metal-induced O_2^- formation could cause to transcriptional activation of genes responsible for antioxidative enzymes. O_2^- formation could be associated with an increased activity of SOD for conversion and parallel increased activity of CAT. Furthermore, Mittler [37] reported that increased level of antioxidants has a pivotal role in deteriorating the ROS activity, thus plants could be able to maintain their physiological functions under the stress environment. Along with primary defense mechanism, plants also activated their non-enzymatic antioxidant system named as phenolics and proline as a result of biotic and abiotic stresses, including heavy metal

toxicity [38]. Stress induced proline accumulation can reduce photochemical activity losses and the production of free radicals in the thylakoid membrane of the chloroplast [39]. Thus, proline contributed to arrest photo inhibitory damage in plants. Many plants accumulate proline at higher concentration in response to toxic concentrations of heavy metals [40]. Some researchers conclude that proline accumulation is not related to protection against metal stress [41], is just a symptom of injury. On the contrary, it has been suggested that proline might have an adaptive role related to survival of plants against heavy metal toxicity [42]. Zengin and Munzuroglu [43] showed that copper and mercury toxicity increased the proline content of the leaves of bean (*Phaseolus vulgaris* L.) seedlings. A similar observation was also recorded in our experiment that there were a positive correlation between metal toxicity and proline accumulation in callus cultures of *Digitalis* species. Therefore, we may conclude that proline may have a protective an adaptive role against stress conditions.

Studies suggested that H_2O_2 contents increased under both biotic and abiotic stresses induced expression of phenyl ammonia lyase (PAL) accompanied with the de novo synthesis of phenolics [44]. Phenolic compounds have antioxidant action because they are particularly bind iron and copper owing to their high tendency to chelate metals [45]. The content of free phenols was found to increase in two lines of wheat (*T. aestivum*) and root cultures of *Lupinus albus* L. with increasing Cu and Hg concentration in the medium, respectively. [46, 47]. In our studies, the increase in phenolic levels observed in the callus cultures of *Digitalis* species to reduce the oxidative stress caused by Cu and Hg.

5. CONCLUSION

This study demonstrated the role of heavy metals in the stimulated production of cardenolides in the callus cultures of *Digitalis* species. The productivity of cardenolides was found to be dependent on types of species and two elicitors. The present study has established that $CuSO_4$ is a better elicitor than $HgCl_2$ for cardenolide production from *Digitalis* callus cultures. This study would help to intentionally manipulate elicitation strategy to improve the yield of cardenolides profile in the callus cultures as well as to extend this protocol for large scale production of cardenolides in bioreactor utilizing *Digitalis* species in order to cope up with the demand for cardenolides in international pharmaceutical markets in future. Our results also showed that enzymatic and non-enzymatic antioxidative system are sensitive to these stress factors. Different accumulation trends were detected between individual compounds against applied treatments. We expect that further analysis of cardenolides and antioxidant molecules will provide insights into the regulatory relationships among these molecules and the role of these molecules in the establishment of mechanism for cardenolide production. The results presented in this work regarding the analysis of cardenolides under stress conditions are believed to create a useful base for the future studies in understanding the antioxidant mechanism which can be employed for the improvement of a large scale production of cardenolides.

Acknowledgement

This study was supported by the Abant Izzet Baysal University Research Foundation (Project No: 2010.03.01.341).

Conflict of Interests

Authors declare that there is no conflict of interests.

6. REFERENCES

[1]. Baytop, T. (1999). Türkiye'de Bitkilerle Tedavi. *Istanbul: Nobel Tıp Kitabevi*.

[2]. Newman, R.A., Yang P., Pawlus A.D., & Block K.I. (2008). Cardiac glycosides as novel cancer therapeutic agents. *Molecular Interventions*, 8, 36-49.

[3]. Verma, S.K., Sahin, G., Yucesan, B., Sahbaz, N., Eker, I., Gurel, S., & Gurel, E. (2012). Direct somatic embryogenesis from hypocotyl segments of *Digitalis trojana* Ivan and subsequent plant regeneration. *Industrial Crops and Products*, 40, 76-80.

[4]. Radman, R., Saez, T., Bucke, C., & Keshavarz, T. (2003). Elicitation of plants and microbial cell systems. *Biotechnology and Applied Biochemistry*, 37, 91-102.

[5]. Zhao, J., Davis, L.C., & Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances*, 23, 283-333.

[6]. Kim, O.T., Bang, K.H., Kim, Y.C., Hyun, D.Y., Kim, M.Y., & Cha, S.W. (2009). Upregulation of ginsenoside and gene expression related to triterpene biosynthesis in ginseng hairy root cultures elicited by methyl jasmonate. *Plant Cell Tissue and Organ Culture*, 98, 25–33.

[7]. Lin, C.C., Chen, L.M., Liu, Z.H. (2005). Rapid effect of copper on lignin biosynthesis in soybean roots. *Plant Science*, 168, 855-861.

[8]. Maksymiec, W. (2007). Signalling responses in plants to heavy metal stress. *Acta Physiologiae Plantarum*, 29, 177-187.

[9]. Agrawal, V., & Sharma, K. (2006). Phytotoxic effects of Cu, Zn, Cd and Pb on *in vitro* regeneration and concomitant protein changes in *Holarrhena antidysenterica*. *Biologia Plantarum*, 50, 307-310.

[10]. Parmar, G.N., & Chanda, V.S. (2005). Effects of Mercury and Chromium on Peroxidase and IAA Oxidase Enzymes in the Seedlings of *Phaseolus vulgaris*. *Turkish Journal of Biology*, 29, 15-21.

[11]. Shanker, A.K., Cervantes, C., Loza-Tavera, H., & Avudainayagam, S. (2005). Chromium toxicity in plants. *Environment International*, 31, 739-753.

[12]. Swaran, J.S.F. (2009). Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. *Oxidative Medicine and Cellular Longevity*, 2, 191-206.

[13]. Cho, U., & Park, J. (2000). Mercury induced oxidative stress in tomato seedlings. *Plant Science*, 156, 1-9.

[14]. Wiegrebe, H., & Wichtl, M. (1993) High-performance liquid chromatographic determination of cardenolides in *Digitalis* leaves after solid-phase extraction. *Journal of Chromatography A*, 630, 402-407.

[15]. Cingoz, S.G., Verma, S.K., & Gürel, E. (2014). Hydrogen peroxide-induced antioxidant activities and cardiotonic glycoside accumulation in callus cultures of endemic *Digitalis* species. *Plant Physiology and Biochemistry*, 82, 89-94.

[16]. Sun, Y., Oberley, L.W., & Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. *Clinical Chemistry*, 34, 497-500.

[17]. Lartillot, S., Kedziora, P., & Athias, A. (1988). Purification and characterization of a new fungal catalase. *Preparative Biochemistry and Biotechnology*, 18, 241-246.

[18]. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the Folin Phenol Reagent. *The Journal of Biological Chemistry*, 193, 265-75.

[19]. Marigo, G. (1973). Sur une méthode de'fractionnement et d' estimation des composés phénoliques chez les vegétaux. *Analusis*, 2, 106-110.

[20]. Bates, L.S., Waldren, R.P., & Teare, I.D. (1973). Rapid determination of free proline for water-stress studies. *Plant Soil*, 39, 205-207.

[21]. Koricheva, J., Larssson, S., Haukioja, E., & Keinanen, M. (1998). Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. *Okios*, 83, 121-226.

[22]. Eisenbeiß, M., Kreis, W., & Reinhard E. (1999). Cardenolide biosynthesis in light- and dark-grown *Digitalis lanata* shoot cultures. *Plant Physiology and Biochemistry*, 37,13–23.

[23]. Gavidia, I., Del Castillo Agudo, L., & Perez-Bermudez, P. (1996). Selection and long-term cultures of high-yielding *Digitalis obscura* plants: RAPD markers for analysis of genetic stability. *Plant Science*, 121, 197-205.

[24]. Gavidia, I., & Perez-Bermudez, P. (1997). *Digitalis obscura* cardenolides effect of macronutrient concentration and N source on growth and productivity of shoot–tip cultures. *Phytochemistry*, 46, 273-238.

[25]. Roca-Perez, L., Boluda, R., & Perez-Bermudez, P. (2004). Soil–plant relationships, micronutrient contents and cardenolide production L. in natural populations of *Digitalis obscura*. *Journal of Plant Nutrition and Soil Science*,167, 79-84.

[26]. Stuhlfauth, T., Klug, K., & Fock, H.P. (1987). The production of secondary metabolites by *Digitalis lanata* during CO_2 enrichment and water stress. *Phytochemistry*, 26, 2735-2739.

[27]. Brugidou, C., Jacques, M., Cosson, L., Jarreau, F.X., & Ogerau, T. (1988). Growth and digoxin content in *Digitalis lanata* in controlled conditions and natural environment. *Planta Medica*, 54, 262–265.

[28]. Sahin, G., Verma, S.K., & Gürel, E. (2013). Calcium and magnesium elimination enhances accumulation of cardenolides in callus cultures of endemic *Digitalis* species of Turkey. *Plant Physiology and Biochemistry*, 73, 139-143.

[29]. Palacio, L., Baeza, C., Cantero, J.J., Cusido´, R., & Goleniowsi, M.E. (2008). *In Vitro* propagation of "Jarilla" (*Larrea divaricata* CAV.) and secondary metabolite production. *Biological and Pharmaceutical Bulletin*, 31, 2321-2325.

[30]. Maksymiec, W., & Krupa, Z. (2006). The effects of short term exposition to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*. *Environmental and Experimental Botany*, 57, 187-194.

[31]. Smith, R.A., Drummond, S., Haines, A., Porter, J.R., & Hock, R.S (2001). Induction of umbelliferone in sweet potato cell suspension culture using mercuric chloride. *Biotechnological Letters*, 23, 1397-1400.

[32]. Korsangruang, S., Soonthornchareonnon, N., Chintapakorn, Y., Saralamp, P., & Prathanturarug, S. (2010). Effects of abiotic and biotic elicitors on growth and isoflavonoid accumulation in *Pueraria candollei* var. *candollei* and *P. Candollei* var. *mirifica* cell suspension cultures. *Plant Cell Tissue and Organ Culture*, 103, 333-342.

[33]. Perez-Bermudez, P., Garcıa, A.A.M., Tunon, I., & Gavidia, I. (2010). *Digitalis purpurea* P5βR2, encoding steroid 5β-reductase, is a novel defense-related gene involved in cardenolide biosynthesis. New Phytologist, 185, 687–700.

[34]. Hung, S.H., Yu, C.W., Lin, C.H. (2005). Hydrogen peroxide functions as a stress signal in plants. *Botanical Bulletin Academia Sinica*, 46, 1-10.

[35]. Ikeda, Y., Fujii, Y., & Yamazaki, M. (1992). Determination of Lanatoside C and Digoxin in *Digitalis lanata* by HPLC and its Application to Analysis of the Fermented Leaf Powder. *Journal of Natural Products*, 55, 748-752.

[36]. Romero-Puertas, M.C., Perazzolli, M., Zago, E.D., & Delledonne, M. (2004). Nitric oxide signalling functions in plant-pathogen interactions. *Cellular Microbiology*, 6, 795-803.

[37]. Mittler R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, 7, 405-410.

[38]. Chen, C.T., Chen, L.M., Lin, C.C., & Kao, C.H. (2001). Regulation of Proline Accumulation in Detached Rice Leaves Exposed to Excess Copper. *Plant Science*, 160, 283-290.

[39]. Alia, A., Saradhi, P.P., & Mohanty P. (1991). Proline enhances primary photochemical activities in isolated thylakoid membranes of *Brassica juncea* by arresting photo inhibitory damage. *Biochemical and Biophysical Research Communications*, 181, 1238-1244.

[40]. Schat, H., Sharma, S.S., & Vooijs, R. (1997). Heavy metal-induced accumulation of free proline in metal-tolerant and a nontolerant ecotype of *Silene vulgaris*. *Physiologia Plantarum*, 101, 477-482.

[41]. Lutts, S., Kinet, J.M., & Bouharmont, J. (1996). Effects of various salts and of mannitol on ion and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) callus cultures. *Journal of Plant Physiology*, 149, 186-195.

[42]. Siripornadulsil, S., Train, S., Verma, D.P.S., & Sayre, R.T. (2002). Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *Plant Cell*, 14, 2837-2847.

[43]. Zengin, F.K., & Munzuroğlu, Ö. (2005). Effects of some heavy metals on content of chlorophyll, proline and some antioxidant chemicals in bean (*Phaseolus vulgaris* L.) seedlings. *Acta Biologica Cracoviensia Series Botanica*, 47, 157-164.

[44]. Grant, J.J., Yun, B.W., & Loake, G.J. (2000). Oxidative burst and cognate redox signaling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *The Plant Journal*, 24, 569-582.

[45]. Jung, C.H., Maeder, V., Funk, F., Frey, B., Sticher, H., & Frosserd, E. (2003). Release of phenols from *Lupinus albus* L. roots exposed to Cu and their possible role in Cu detoxification. *Plant Soil*, 252, 301-312.

[46]. Ganeva, G., & Zozikova, E. (2007). Effect of increasing Cu2+ concentrations on growth and content of free phenols in two lines of wheat (*Triticum aestivum*) with different tolerance. *General and Applied Plant Physiology*, 33, 75-82.

[47]. Esteban, E., Morenoa, E., Penalosa, J., Cabrero, J.I., Millan, R., & Zornoza, P. (2008). Short and long-term uptake of Hg in white lupin plants: Kinetics and stress indicators. *Environmental and Experimental Botany*, 62, 316-322.